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(54) Title: HUMAN PLATELET-SPECIFIC ANTIBODIES

(57) Abstract

Human monoclonal immunoglobulin and immunoglobulin fragments that are specific for blood platelets are described. The immunoglobulin, or fragment thereof, are, preferably, specific for the glycoprotein IIb/IIIa receptor in its complexed form. These antibodies block ligand binding to the receptor, thereby preventing platelet aggregation which has been implicated in the formation of thrombi. These immunoglobulines are useful in anti-thrombotic therapy alone or in conjunction with thrombolytic agents, and in scintigraphic imaging of thrombi.

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HUMAN PLATELET-SPECIFIC ANTIBODIES

Description

Background of the Invention

Platelet aggregation is an essential event in the formation of blood clots. Under normal circumstances, blood clots serve to prevent the escape of blood cells from the vascular system. However, during certain disease states (e.g., myocardial infarction), clots can restrict or totally prevent blood flow, resulting in cellular necrosis.

Heart attack patients are typically treated with thrombolytic agents such as tissue plasminogen activator or streptokinase, which dissolve the fibrin component of clots. A major complication associated with fibrinolysis is reocclusion based on platelet aggregation which can result in further heart damage. Since glycoprotein IIb/IIIa (GPIIb/IIIa) receptors are known to be responsible for platelet aggregation, reagents that block these receptors are expected to reduce or prevent reocclusion following thrombolytic therapy and to accelerate the rate of thrombolysis.

One approach to blocking platelet aggregation involves monoclonal antibodies specific for GPIIb/IIIa receptors. A murine monoclonal antibody, designated 7E3, that inhibits platelet aggregation and appears useful in the treatment of human thrombotic diseases is described in published European Patent Application Nos. 205,207 and 206,532. Murine antibodies have characteristics that may severely limit their use in human therapy. They are foreign proteins, which may elicit immune reactions that

reduce or destroy their therapeutic efficacy and/or evoke allergic or hypersensitivity reactions in patients. The need for readministration of such therapeutic modalities in thromboembolic disorders increases the likelihood of these types of immune reactions.

Summary of the Invention

This invention pertains to human platelet-specific monoclonal antibodies. The antibodies are specific for the GPIIb/IIIa receptor, or other platelet components. These antibodies bind to platelets, and can block platelet aggregation, and thus, are useful as antithrombotic agents, and to prevent or reduce reocclusion following thrombolysis. Human platelet-specific antibodies minimize some of the problems often associated with the immunogenicity of antibodies composed of nonhuman protein.

Detailed Description of the Invention

The present invention relates to human platelet-specific monoclonal antibodies. The antibodies are comprised entirely of human protein. These antibodies target platelet components, such as the GPIIb/IIIa receptor. The antibodies bind to platelets and thereby prevent platelet aggregation and thrombus formation.

The human antibodies invention are specific for platelet surface components. Preferred are specific for platelet GP IIb/IIIa receptors; they bind to the GPIIb/IIIa receptor and block ligand binding to the GPIIb/IIIa receptor complex. The preferred antibodies are specific for the complexed form of GPIIb/IIIa

receptor. However, antibodies can be also specific for either the GPIIb or GPIIIa components. Alternatively, antibodies specific for other platelet antigens can also be employed. For example, human antibodies that bind to platelet granule membrane protein GMP-140 can be used.

In general, platelet-specific antibodies can be prepared by obtaining lymphoid cells from an individual who produces antibody against a platelet antigen. The lymphoid cells are fused to immortalizing cells to produce continuous hybrid cell lines. Hybrid cells producing antibody against the desired platelet antigen are selected and cloned.

In a preferred embodiment, human monoclonal antibody specific for GPIIb/IIIa can be prepared as follows. Since GPIIb/IIIa is normally on all human platelets, humans are 'tolerant' to this heterodimer and do not mount an antibody response to it. Certain rare individuals (e.g. individuals with Glanzmann's thrombasthenia) do not express this complex on their platelets. Individuals who lack GPIIb/IIIa may mount an antibody response when exposed to GPIIb/IIIa. Such exposure would be likely to occur during the course of transfusions that include blood platelets. Transfusion might be employed for a variety of medically justified reasons. In the case of Glanzmann patients it would be used to treat hemorrhages caused by the inability of their defective (i.e. GPIIb/IIIa lacking) platelets to aggregate properly. Following transfusions such individuals would be expected to respond immuno-

logically to the GPIIb/IIIa complex just as they would to any 'foreign' protein. They would mount a B cell response with the appearance of specific antibody in their blood and with an amplification of antigen-specific B cells. A hybridoma capable of secreting human monoclonal antibody specific to the human GPIIb/III complex can be made from B cells from such patients.

An individual is identified who has a serum antibody titer to GPIIb/IIIa. Such an individual might be one who lacks the heterodimer as in Glanzmann's thrombasthenia. This individual after having been exposed to normal (GPIIb/IIIa-containing) platelets as a result of a transfusion would be expected to develop an antibody response. Alternatively, a normal individual might be exposed to GPIIb/IIIa in an immunogenic fashion. This might take the form of repeated transfusions wherein some of the material might become partially denatured and hence more immunogenic or it might occur through the binding of a drug or other substance to the platelets causing modification of surface molecules and eliciting an antibody response. Additionally, individuals with autoimmune disease, such as idiopathic thrombocytopenic purpura might be suitable sources of antigen specific B lymphocytes.

B lymphocytes are obtained from the individual in the form of, for example, spleen, lymph nodes or peripheral blood obtained by venipuncture or pheresis. The lymphoid cells can be enriched by use of a one step gradient such as Ficoll-Hypaque. The recovered cells can be washed to thoroughly remove the gradient material which may be toxic.

Unwanted or undesirable cell populations such as suppressor cells ($CD8^+$) or B cells making an unwanted isotype such as IgM are removed. This may be accomplished by complement mediated lysis, cell sorting using flow cytometry or affinity purification such as 'panning'.

Prior to fusion, the B cells can be stimulated with antigen, lymphokines and/or other mitogenic substances or substances that will induce the B cells to synthesize and secrete antibody.

The appropriately stimulated cells are then fused using polyethylene glycol or other fusogenic agents or devices. The fusion partner is a cell or hybrid of B cell lineage capable of supporting the synthesis and secretion of human antibodies.

Generally, the immortalizing cell line is a tumor cell, which endows the hybridoma with the ability to grow permanently in culture. This ensures a stable culture of antibody-producing hybridoma cells which can produce monoclonal antibodies in a continuous supply. The immortalizing cell may be a plasmacytoma cell, such as a myeloma cell. The myeloma cell can be human, non-human, or a heteromyeloma. Suitable human immortalizing cell lines include the HMMA2.11, HF2 cell line, and the U-266. A heteromyeloma is a myeloma hybrid formed by the fusion of cells of two different species. See Oestberg, U.S. Patent 4,634,664.

The cell fusions are accomplished by standard procedures. See, Kohler and Milstein, Nature (London), 256:495-497 (1975); Olsson and Kaplan, Proc. Natl. Acad. Sci. USA 77:5429 (1980).

The hybridomas are then screened for production of antibodies reactive with platelets or platelet component such as the GPIIb/IIIa receptor. The screening can be accomplished by an enzyme immunoassay. For example, purified GPIIb/IIIa can be bound to a solid phase. The solid phase can then be contacted with hybridoma supernatant and antibody binding to the GPIIb/IIIa-solid phase can be evaluated with enzyme-conjugated anti-human antibody. Hybridomas that secrete reactive antibodies are cloned.

Another method of forming the antibody-producing cells is by viral or oncogenic transformation. For example, human B-lymphocyte which produced a plate-specific antibody may be infected and transformed with a virus, such as the Epstein-Barr virus, to give an immortal antibody-producing cell. See, e.g., Kozbor and Roder (1983) Immunology Today, 4(3):72-79. Or, the B-lymphocyte may be transformed by a transforming gene or gene product.

Monoclonal antibodies are generally produced in large quantities by culturing hybridomas that produce anti-platelet antibody in vitro and isolating the secreted monoclonal antibodies from the cell culture medium.

The human platelet-specific antibodies of this invention are useful as antithrombotic therapeutic agents. The antibodies (or fragments thereof) can be used to inhibit platelet aggregation and thrombus formation. The antibodies can be used in any situation where thrombus formation or reformation is to be prevented. For example, the antibody alone can be used to prevent clotting in post-angioplasty treatment, pulmonary

embolism, deep vein thrombosis and coronary bypass surgery. The antibody can also be administered in conjunction which a thrombolytic agent, such as tissue plasminogen activator, streptokinase, single chain streptokinase, acyl-plasminogen-streptokinase activator complex, urokinase or the mutant variants of tissue plasminogen activator, streptokinase and urokinase, to prevent or reduce reocculusion that can occur after thrombolysis, and to accelerate clot lysis. The antibody or fragment can be administered before, along with, or subsequent to administration of the thrombolytic agent, in an amount sufficient to prevent platelet aggregation, which can result in reocclusion. The antibody is given parenterally, preferably intravenously, in a pharmaceutically acceptable vehicle such as sterile saline. The antibody could be given multiple times or by a controlled release mechanism (e.g., by a polymer or patch delivery system).

During repeat therapy with anti-platelet antibodies drug-induced thrombocytopenia may occur; this may be a result of the body recognizing the antibody-coated platelets as foreign proteins, raising antibodies against them and then clearing them more rapidly than normal. The use of a human anti-platelet antibody may avoid this problem.

The platelet-specific human antibody of this invention is also useful for thrombus imaging. For this purpose, antibody fragments are generally preferred. Antibody fragments such as Fab, Fab' and F(ab')2 can be produced by standard procedures. The fragments can be labeled directly, or through a coupled chelating agent

such as diethylenetriaminepentaacetic acid, with radioisotopes such as ^{131}Iodine, ^{125}Iodine, ^{99m}Technetium or ^{111}Indium to produce radioimmunoscintigraphic agents.

The radiolabeled antibody is administered to a patient suspected of having thrombus. After sufficient time to allow the labeled immunoglobulin to localize at the thrombus site, the signal generated by the label is detected by a photoscanning device such as a gamma camera. The detected signal is then converted to an image of the thrombus. The image makes it possible to locate the thrombus in vivo and to devise an appropriate therapeutic strategy.

The invention is further illustrated by the following examples.

EXEMPLIFICATION

A donor was identified who had been diagnosed as having Glanzmann's thrombasthenia. She had no detectable GPIIb/IIIa on her platelets. She had a transfusion history of over 100 blood transfusions. She had a demonstrable IgG anti-GPIIb/IIIa titer as determined by EIA using purified GPIIb/IIIa as the solid phase.

The donor was lymphopheresed using a Fenwal CS3000 Blood Cell Separator. The cells were collected in an acid-citrate dextrose anticoagulant. A total of 125ml containing 1.6×10^9 cells were obtained. A white cell differential analysis showed 93% lymphocytes. The cells were diluted 1:3 in Hanks' Balanced Salt Solution (HBSS) and layered over Ficoll-Paque. The cells recovered from the interface were washed two times in HBSS. Recovery by

Coulter count was $1.5x10^9$. The cells were pooled into 4 groups (which were processed separately throughout the remainder of the experiment) and each pool was placed in a $75cm^2$ tissue culture flask which had been pre-coated with 50μg/ml of goat anti-human IgM and 50μg/ml of mouse monoclonal anti-CD8 antibodies for 2hrs at 4°C. The cells were allowed to adhere to the antibody coated plates for 45 minutes with occasional gentle agitation at 4°C. At the end of this 'panning' step $1.0x10^9$ cells (Coulter count) were recovered by gently removing the non-adherent cells. The cells were washed with HBSS and each group was seeded at $2x10^6$ cells/ml in 30ml in $75cm^2$ tissue culture flasks (4 flasks/group, a total of 16 flasks). Each 500ml of medium ('modified' alpha MEM) was supplemented with Eagle's nonessential amino acids (100x, 5ml), sodium pyruvate (100mM, 5ml), glutamine (200mM, 5ml), fetal bovine serum (100ml), gentamycin (50mg/ml; 2.5ml), Pokeweed Mitogen (Gibco, 0.25ml), and GPIIb/IIIa adsorbed to fumed silica (final concentration of GPIIb/IIIa, 1μg/ml). The cells were incubated for 4 days at 37°C in a humidified atmosphere of 5% CO_2 in air.

On the fourth day, the stimulated lymphocytes were fused with the human myeloma analogue HMMA 2.11tg/o. The lymphocytes in each group were mixed with an equivalent number ($1x10^9$) of HMMA cells. The cells were washed 2 times in HBSS, pH 7.8 and the resulting pellets were very slowly resuspended in 1.5ml of polyethylene glycol (PEG) (46% w/v in HBSS pH 7.8, m.w. 8,000) over a period of 3 minutes with constant agitation. The fused cells were then allowed to remain in the PEG for an additional one minute. The cells were then slowly resuspended in HBSS

containing 5% fetal bovine serum. Ten ml were added over a period of 3 minutes with constant agitation. Another 10ml were added in the next 1 minute. The cells were then centrifuged and resuspended in 'modified' alpha MEM supplemented as above except that Pokeweek Mitogen and silica adsorbed GPIIb/IIIa were omitted and HAT (hypoxanthine, aminoipterin, thymidine) was added. Each fusion was then distributed in 75cm^2 flasks (5), 50ml/flask, 1x10^6 hybrid equivalents/flask. The cells were incubated for 48 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. They were then redistributed into 100 flat bottom 96 well tissue culture plates and further incubated until they were ready to be screened for antibody production.

Initial screening consisted of identifying those hybrids which secreted IgG antibodies which bound to human platelet derived GPIIb/IIIa. Purified GPIIb/IIIa was allowed to adhere to 96 well polystyrene EIA plates (Costar) at a concentration of 2μg/ml overnight at 4°C. The plates were washed, blocked with 3% bovine serum albumin-1% normal goat serum for one hour, supernate was added and incubated for one hour, washed and then incubated with goat anti-human IgG conjugated to horseradish peroxidase, incubated for one hour and then developed with o-phenylenediamine. From the four fusions two hybrids were obtained which secreted antibodies detectable by this assay. The two positive hybrids designated Gimmel 51F11 and Gimmel 51G10 were subcultured, cloned and cryopreserved in liquid nitrogen.

Equivalents

Those skilled in the art will recognized, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims

1. A human monoclonal platelet-specific antibody.

2. A human monoclonal antibody of Claim 1 that specifically binds the glycoprotein IIb/IIIa receptor complex of platelets.

3. An antigen-binding fragment of the human monoclonal antibody of Claim 1.

4. A radiolabeled antigen binding fragment of Claim 3.

5. A human monoclonal platelet-specific antibody fragment Fab, Fab' or $F(ab')_2$.

6. A human, monoclonal antibody fragment of Claim 5 which is radiolabeled.

7. A human monoclonal antibody of Claim 6 wherein the radiolabel is selected from the group consisting of $^{99m}_{TC}$ $^{111}_{In}$, $^{125}_{I}$ and $^{131}_{I}$.

8. A hybridoma that produces human monoclonal anti-platelet antibody, the hybridoma produced by the fusion of a human lymphocyte from an individual immunized against platelets and a human lymphoblastoid cell.

9. A hybridoma of Claim 8 wherein the lymphocyte is obtained from an individual immunized against the gp IIb/IIIa receptor.

10. A hybridoma of Claim 8, wherein the lymphocyte is a peripheral blood lymphocyte.

11. A method of producing a human platelet-specific monoclonal antibody, comprising:
a. obtaining lymphoid cells from an individual who produces antibody against a platelet antigen;
b. fusing the lymphoid cells with an immobilizing cell to produce a hybrid cell; and
c. selecting and cloning hybrid cells that produce antibody against the platelet antigen.

12. A method of Claim 11, wherein the platelet antigen is GPIIb/IIIa.

13. A method of Claim 12, wherein the individual has Glanzmann's thrombasthenia.

14. A method of antithrombotic therapy, comprising administering to a patient having a thrombus, or at risk of thrombus formation, an anti-thrombotic amount of a human monoclonal antibody or antibody fragments specific for blood platelets.

15. A method of Claim 14 wherein the antibody or fragments are specific for the GPIIb/IIIa receptor.

16. A method of Claim 15 wherein the antibody fragment is an Fab, Fab' or $F(ab')_2$ fragment.

17. A method of antithrombotic therapy, comprising administering to a patient having a thrombus or at risk for thrombus formation, a thrombolytic agent and a human monoclonal antibody or antibody fragment specific for platelets.

18. A method of Claim 17, wherein the human monoclonal antibody or antibody fragment is administered along with or subsequent to administration of the thrombolytic agent.

19. A method of Claim 17, wherein the thrombolytic agent is tissue plasminogen activator, streptokinase, single chain streptokinase, acyl-plasminogen-streptokinase activator complex, urokinase or the mutant variants of tissue plasminogen activator, streptokinase and urokinase.

20. A method of Claim 17, wherein the human monoclonal antibody or fragment is specific for glycoprotein IIb/IIIa.

21. A method of Claim 17, wherein the antibody fragment is an Fab, Fab' or $F(ab')_2$ fragment.

22. A method of thrombus imaging, comprising:

a. administering to an individual suspected of having a thrombus, a radiolabeled human

platelet-specific human antibody or fragment thereof;

b. allowing the antibody or antibody-fragment to accumulate at a thrombus site;

c. detecting the signal generated by the radiolabel by means of a photo scanning device; and

d. converting the detected signal to an image of the thrombus.

23. A method of Claim 22, wherein a Fab, Fab' or $F(ab')_2$ fragment is administered.

24. A method of Claim 22, wherein the radiolabel is selected from the group consisting of ^{99m}Tc, ^{111}In, ^{125}I and ^{131}I.

25. A method of Claim 22, wherein the platelet-specific antibody or fragment is specific for the glycoprotein IIb/IIIa receptor.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/05418

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 39/395, 49/02, C 12 P 21/08, C 12 N 5/22

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System [8]	Classification Symbols
IPC5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [9]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Blood, Vol. 70, No. 1, July 1987, D.J. Nugent et al: "A human monoclonal autoantibody recognizes a neoantigen on glycoprotein IIIA expressed on stored and activated platelets ", see page 16 - page 22 see especially page 16	1,2
Y		3-13,22-25
Y	J. Clin. Invest., Vol. 81, April 1988, T. Y. Herman et al: "Monoclonal antibody against the platelet glycoprotein (GP) IIb/IIIa receptor prevents coronary artery reocclusion after reperfusion with recombinant tissue-type plasminogen activator in dogs ", see page 1284 - page 1291	1-3

* Special categories of cited documents: [14]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29th March 1990	12.04.90

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	F.W. HECK

III. DOCUMENTS CONSIDERED T BE RELEVANT (C NTINUED FROM THE SEC ND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	J. Clin. Invest., Vol. 81, January 1988, S.R. Hanson et al: "Effects of monoclonal antibodies against the platelet glycoprotein IIb/IIIa complex on thrombosis and hemostasis in the baboon ", see page 149 - page 158	1-3
Y	EP, A2, 0206533 (THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK) 30 December 1986, see the whole document	1-3
A	EP, A2, 0206532 (THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK) 30 December 1986, see the whole document	1-3
Y	British Medical Bulletin, Vol. 40, No. 3, 1984, K. Sikora: "Human monoclonal antibodies ", see page 209 - page 212	1-13
Y	British Medical Journal, Vol. 293, December 1986, A.M. Peters et al: "Imaging thrombus with radiolabelled monoclonal antibody to platelets ", see page 1525-27	1-7,22-25
Y	EP, A2, 0205270 (THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK) 17 December 1986, see the whole document	1-7,22-25

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 14-21 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv)

Method for treatment of the human or animal body by means of surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 89/05418

SA 33565

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 28/02/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0206533	30/12/86	JP-A- 62029995	07/02/87
EP-A2- 0206532	30/12/86	JP-A- 62030728	09/02/87
EP-A2- 0205270	17/12/86	JP-A- 62076463	08/04/87